

University of Groningen

Quaternary Structure of SecA in Solution and Bound to SecYEG Probed at the Single Molecule Level

Kusters, Ilja; van den Bogaart, Geert; Kedrov, Alexej; Krasnikov, Victor; Fulyani, Faizah; Poolman, Bert; Driessen, Arnold J. M.

Published in:
Structure

DOI:
[10.1016/j.str.2010.12.016](https://doi.org/10.1016/j.str.2010.12.016)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2011

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Kusters, I., van den Bogaart, G., Kedrov, A., Krasnikov, V., Fulyani, F., Poolman, B., & Driessen, A. J. M. (2011). Quaternary Structure of SecA in Solution and Bound to SecYEG Probed at the Single Molecule Level. *Structure*, 19(3), 430-439. <https://doi.org/10.1016/j.str.2010.12.016>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Quaternary Structure of SecA in Solution and Bound to SecYEG Probed at the Single Molecule Level

Ilja Kusters,¹ Geert van den Bogaart,^{2,4} Alexej Kedrov,¹ Victor Krasnikov,³ Faizah Fulyani,^{1,2} Bert Poolman,² and Arnold J.M. Driessen^{1,*}

¹Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, and Zernike Institute for Advanced Materials

²Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, Netherlands Proteomics Centre, and Zernike Institute for Advanced Materials

University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

³Department of Optical Condensed Matter Physics, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

⁴Present address: Max Planck Institute for Biophysical Chemistry, Department of Neurobiology, Am Fassberg 11, Göttingen D-37077 Germany

*Correspondence: a.j.m.driessen@rug.nl

DOI 10.1016/j.str.2010.12.016

SUMMARY

Dual-color fluorescence-burst analysis (DCFBA) was applied to measure the quaternary structure and high-affinity binding of the bacterial motor protein SecA to the protein-conducting channel SecYEG reconstituted into lipid vesicles. DCFBA is an equilibrium technique that enables the direct observation and quantification of protein-protein interactions at the single molecule level. SecA binds to SecYEG as a dimer with a nucleotide- and preprotein-dependent dissociation constant. One of the SecA protomers binds SecYEG in a salt-resistant manner, whereas binding of the second protomer is salt sensitive. Because protein translocation is salt sensitive, we conclude that the dimeric state of SecA is required for protein translocation. A structural model for the dimeric assembly of SecA while bound to SecYEG is proposed based on the crystal structures of the *Thermotoga maritima* SecA-SecYEG and the *Escherichia coli* SecA dimer.

INTRODUCTION

Protein secretion in bacteria is mediated by a multiprotein complex termed translocase. It consists of the protein-conducting channel SecYEG and the motor protein SecA (for review, see Driessen and Nouwen, 2008). Posttranslational protein translocation starts with the binding of the mature region of the secretory protein (preprotein) by the molecular chaperone SecB (Hartl et al., 1990), whereupon it is targeted to the SecYEG-bound SecA (Fekkes et al., 1998). Upon ATP binding to SecA, the signal sequence of the preprotein inserts into the SecYEG channel, and SecB is released. Next, SecA mediates the stepwise translocation of the preprotein through multiple cycles of ATP hydrolysis (Economou and Wickner, 1994; Schiebel et al., 1991; van der Wolk et al., 1997).

The functional oligomeric state of SecA during the protein translocation cycle is still a matter of controversy. In solution, SecA forms a homodimer and is expected to exist mainly as a dimer in the cytosol (Akita et al., 1991; Woodbury et al., 2002). The monomer-dimer equilibrium can shift toward the monomeric form in the presence of certain lipids (Benach et al., 2003; Or et al., 2002), detergents (Benach et al., 2003; Or et al., 2002), or synthetic signal peptides (Musial-Siwiek et al., 2005; Or et al., 2002), although signal peptides also have been shown to induce oligomerization of SecA (Benach et al., 2003). When bound to lipids, SecA is mainly dimeric and can be monomerized by the addition of nucleotides (Bu et al., 2003). Because SecYEG was not present in any of these studies, it is not clear if these observations are functionally relevant for SecA when actively engaged in preprotein translocation. However, these studies clearly demonstrate the sensitive nature of the monomer-dimer equilibrium of SecA in solution.

Detergent-solubilized SecYEG has been used to determine the oligomeric state of SecYEG-bound SecA, using native gel electrophoresis and size exclusion chromatography. The functional oligomeric state of SecYEG is not known, but it was suggested to be monomeric (Bessonneau et al., 2002; Tziatzios et al., 2004), dimeric (Bessonneau et al., 2002; Mori et al., 2003; Tziatzios et al., 2004; van der Sluis et al., 2002; Veenendaal et al., 2001), and tetrameric (Manting et al., 2000; Meyer et al., 1999). SecA can bind both as monomer or dimer to SecYEG-dimers in detergent solution when the latter is stabilized by crosslinking or by an antibody (Duong, 2003; Tziatzios et al., 2004). Only monomeric SecA was found on detergent-solubilized SecYEG when a preprotein was trapped in the pore prior to membrane solubilization (Duong, 2003). Because detergents affect the monomer-dimer equilibrium of SecA (Benach et al., 2003; Or et al., 2002) and because SecYEG is not in its native membrane environment, the exact functional implications of these observations have also remained obscure. The experimental conditions, in particular the SecA concentration, seem to be crucial and may have led to conflicting results on the oligomeric state of SecA bound to SecYEG. When SecA was used at a concentration far below its physiological concentration (5 nM compared to 8 μ M), no dimeric SecA was found by chemical

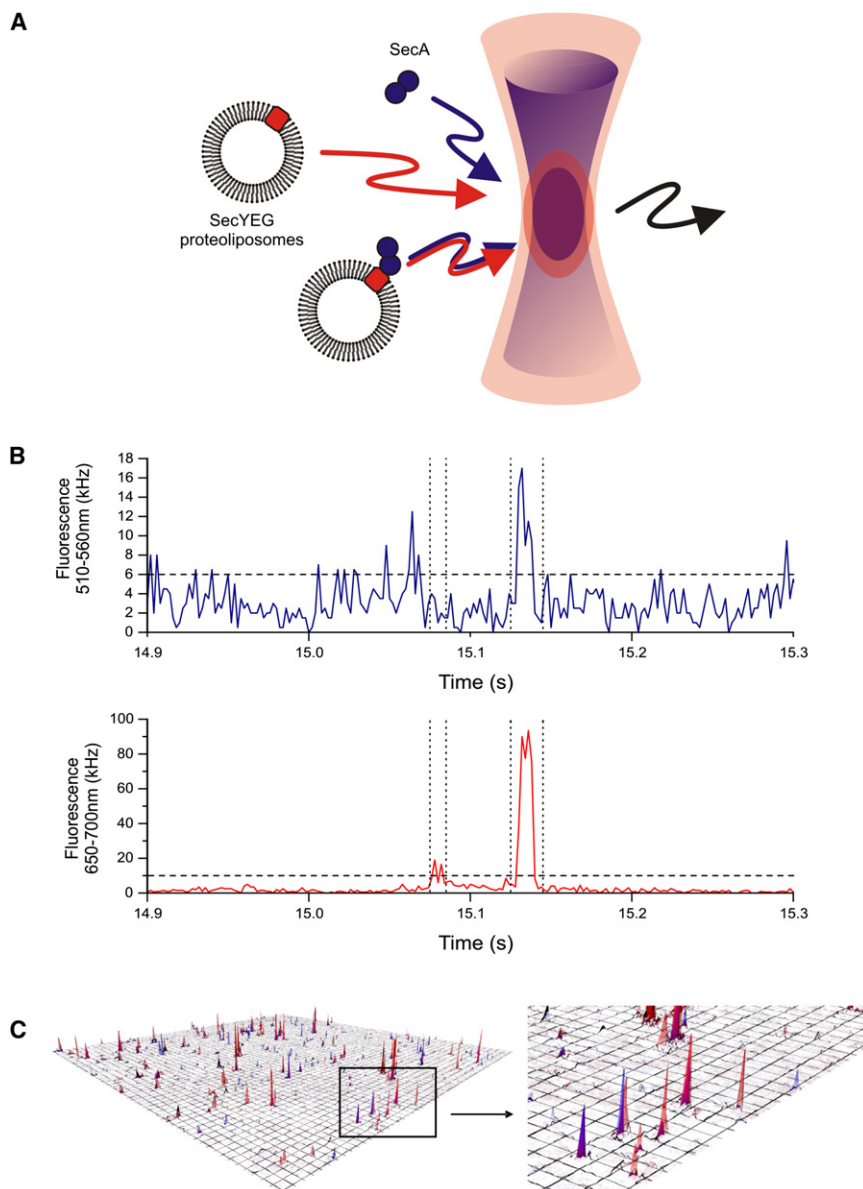


Figure 1. Principle of DCFBA of SecA-SecYEG Binding

(A) Schematic of the principle of DCFBA. Complexes of the blue fluorophore-labeled motor protein SecA and liposome reconstituted with the red fluorophore-labeled SecYEG co-migrate through the two aligned laser foci of a dual-color LSCM.

(B) Recording of fluorescent bursts resulting from proteoliposomes containing fluorescently labeled SecYEG (lower panel; red) and SecA, labeled with a spectrally separated fluorophore (upper panel; blue), passing the detection volumes of a dual-color LSCM. Binding of SecA to its membrane receptor SecYEG leads to co-migration and overlap of the discrete fluorescent bursts. The ratio of the SecA/SecYEG peak surface represents a direct measure of the relative binding stoichiometry. Peaks are selected for analysis by use of noise offsets (dashed lines).

(C) IsDCFBA, height field plot of confocal images of SecA-FM (blue peaks) and SecYEG-Atto647N containing proteoliposomes (red peaks). Peak height represents the fluorescence intensity.

strongly shifted toward the monomer showed a very low translocation activity, or no activity at all (Jilaveanu et al., 2005; Or et al., 2002, 2005; Randall et al., 2005). On the other hand, chemically crosslinked SecA dimers have been shown to be fully active in protein translocation (de Keyzer et al., 2005; Jilaveanu and Oliver, 2006). Furthermore, heterodimers formed from active and nonactive SecA monomers were completely inactive (Driessen, 1993). These studies suggest that dimeric SecA is involved at least in an initial step of protein translocation. Overall, it seems that SecA can bind as a monomer as well as a dimer to SecYEG.

Here, we present an equilibrium method to determine the oligomeric state of proteins when bound to their membrane receptor in a dynamic *in vitro*

system. This method, dual-color fluorescence-burst analysis (DCFBA) was recently employed to determine the pore size of the mechanosensitive channel of large conductance MscL (van den Bogaart et al., 2007a) and the pore-forming mechanism of the antimicrobial peptide melittin from bee venom (van den Bogaart et al., 2007b) (for DCFBA review see van den Bogaart et al., 2008). Now, we have used DCFBA to quantitatively measure the binding of SecA to membrane-reconstituted SecYEG at low nanomolar concentration, and to monitor the oligomeric state of SecA when functionally involved in preprotein translocation. The method is based on a quantitative coincidence analysis of the fluorescent bursts that reside from single liposomes containing fluorescently labeled SecYEG and SecA labeled with a second, spectrally separated fluorophore diffusing through the focal volume of a confocal microscope (Figure 1A). Our data show that dimeric SecA is driving protein translocation.

crosslinking to bind liposome-reconstituted SecYEG (Or et al., 2002). In contrast, dimeric SecA was detected to bind inner membrane vesicles (IMVs), as shown by chemical crosslinking (de Keyzer et al., 2005; Jilaveanu et al., 2005) and surface plasmon resonance (SPR) (de Keyzer et al., 2005). Recently, SecA has been crystallized bound to SecYEG in the presence of ADP-BeFx, a structural analog of an intermediate state of ATP hydrolysis (Zimmer et al., 2008). This structure in which both SecA and SecYEG are monomeric may represent a specific intermediate in the catalytic cycle, but because detergent and high salt were present throughout the crystallization, the conditions employed will favor the dissociation of the SecA dimer.

The oligomeric state of translocation-engaged SecA has also been investigated by activity assays, e.g., the *in vitro* preprotein translocation and preprotein-stimulated SecA translocation ATPase. Mutants of SecA with a monomer-dimer equilibrium

The oligomeric state of translocation-engaged SecA has also been investigated by activity assays, e.g., the *in vitro* preprotein translocation and preprotein-stimulated SecA translocation ATPase. Mutants of SecA with a monomer-dimer equilibrium

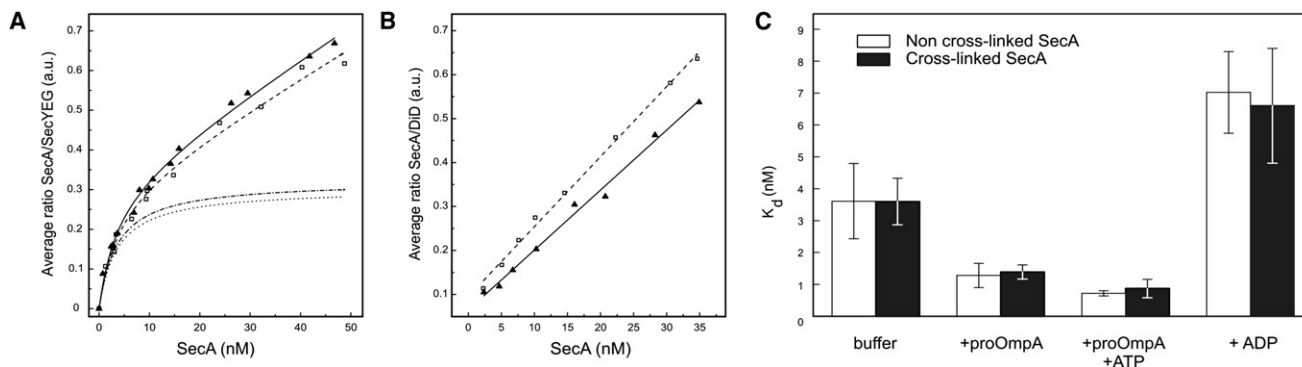


Figure 2. Saturable Binding of SecA to SecYEG

(A) Binding of SecA-FM to SecYEG-Atto647N containing proteoliposomes. The average ratio of SecA/SecY fluorescence was calculated using DCFBA (Equation 1) yielding an arbitrary unit for the binding stoichiometry. Crosslinked SecA-FM (triangles) and non-crosslinked SecA-FM (squares) bind with identical ratios. Data points were fitted using the Hill equation (straight and dashed lines), and the linear nonsaturable binding parameter was subtracted from the fit (dotted lines). (B) Binding of SecA-FM to empty liposomes composed of *E. coli* lipid extract supplemented with 10^{-4} molar percentage (%) of the fluorescent lipid analog DiD. The linear increase of the average ratio SecA/DiD indicates that SecA binds nonspecifically to the lipid membrane. (C) Binding affinities of crosslinked and non-crosslinked SecA to SecYEG in the presence or absence of translocation ligands. Error bars show SD.

See also Figures S1–S4.

RESULTS

SecA Binding to SecYEG and Liposomes Monitored by DCFBA

DCFBA is a fluorescence correlation spectroscopy method wherein the fluorescent bursts that reside from fluorescently labeled proteins that diffuse through the focal volume of a confocal microscope coincide with those from another protein labeled with a second spectrally well-separated fluorophore (Figure 1A). We determined the binding stoichiometry of SecA to SecYEG using DCFBA and employing a dual-color laser-scanning confocal microscope (LSCM) with 488 and 633 nm lasers for excitation of fluorescein maleimide (FM) and Atto647N simultaneously. The pinholes and laser beams are spatially aligned to a high degree of overlap of the two detection volumes allowing the simultaneous detection of co-migrating fluorophores with single molecule resolution (Figure 1B). To employ the method to the protein translocase, the translocation pore SecY_{C295}EG was labeled with the fluorescent probe Atto647N (647 and 669 nm for excitation and emission, respectively) at the unique cysteine position C295 and reconstituted into 100 nm sized liposomes composed of *E. coli* lipids. SecA was labeled with FM (494/521 nm) at its native cysteine residues. To determine the SecA binding to SecYEG, SecA-FM was incubated with the proteoliposomes bearing SecYEG-Atto647N (SecYEG-Atto-PL), and the fluorescent bursts resulting from SecYEG-Atto-PL and SecA-FM passing through the confocal volumes of the two aligned laser beams were recorded using the scanning mode of the microscope (Figure 1C). This allowed identifying the overlap of the fluorescent bursts from SecYEG and SecA (Figure 1B) also at very low SecA concentrations when SecA-SecYEG complexes are only infrequently encountered. Thus, the ratio (c) of SecA over SecYEG could be calculated for each individual burst (*i*) from the overlapping fluorescent bursts using a simple division:

$$C_i = \frac{\int_{t_1}^{t_2} I_{\text{SecA}} dt}{\int_{t_1}^{t_2} I_{\text{SecYEG}} dt}, \quad (1)$$

where I_{SecA} and I_{SecYEG} are the fluorescence intensities of the SecA-FM and SecYEG-Atto647N channels, respectively. These are above noise offsets between times t_1 and t_2 . The average ratio (c) of SecA-FM over SecY_{C295}EG-Atto647N derived from the overlapping fluorescent bursts is an arbitrary unit for the binding stoichiometry of SecA to SecYEG. This ratio was determined as a function of the concentration of SecA-FM, and this resulted in a binding curve (Figure 2A, dashed line). As a control for binding of SecA-FM to lipids (Benach et al., 2003; Or et al., 2002), we used empty liposomes composed of *E. coli* lipids supplemented with the fluorescent lipid analog DiD (Figure 2B, dashed line). The binding of SecA to the empty liposomes increased with the concentration, evident as a linear, nonsaturable increase of the average ratio SecA/DiD. The data from the binding experiments of SecA to SecYEG were fitted using the Hill equation, including a linear parameter to account for this background binding to empty lipids. The obtained dissociation constant (K_d) of 3.6 ± 1.2 nM is in good agreement with the values obtained in earlier studies using SPR (2 nM) (de Keyzer et al., 2003). This result validates DCFBA as a technique to study protein binding and confirms the high binding affinity of SecA to membrane-embedded SecYEG.

SecA Is Dimeric during the Rate-Limiting Step of Protein Translocation

Previously, chemically crosslinked SecA dimers were shown to be fully active in protein translocation (de Keyzer et al., 2005; Jilaveanu and Oliver, 2006). Crosslinking is conveniently realized by oxidation of the C-terminal cysteines and forces SecA to stay dimeric throughout the protein translocation reaction without comprising its activity (de Keyzer et al., 2005). The three C-terminal cysteines of SecA are coordinated by a Zn²⁺ ion in a zinc finger-like motif. In the presence of an excess of zinc, these cysteines are protected against labeling with the fluorescent-alkylating agent FM, whereas labeling can occur at a cysteine at position 98. After removal of Zn²⁺ by the chelator EDTA, FM-labeled SecA was chemically crosslinked into a stable

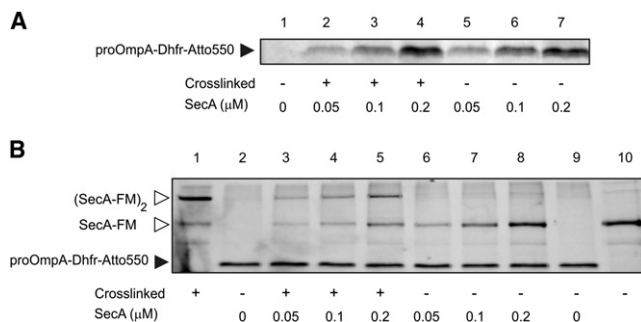


Figure 3. Functional Crosslinked SecA Dimer

(A) In vitro protein translocation with crosslinked SecA. Translocation of fluorescently labeled proOmpA-Dhfr into urea-treated IMVs was assayed with limiting concentrations of SecA-FM. Equal amounts of proOmpA-Dhfr (black arrow) were translocated by both cross and non-crosslinked SecA. The SecA concentration is shown for the monomer.

(B) Chemically crosslinked SecA dimers are stable throughout the translocation reaction. Fractions of the completed in vitro protein translocation reactions shown in (A) were run on SDS-PAGE under nonreducing conditions without prior protease treatment. Covalently crosslinked SecA remained dimeric [(SecA-FM)₂] after the reaction, whereas non-crosslinked SecA dimers dissociated in SDS and migrate as monomers (SecA-FM, white arrows). Equal amounts of proOmpA-Dhfr-Atto550 were used for the translocation reactions (black arrow).

dimer by oxidation with copper phenanthroline (CuPhe) (de Keyser et al., 2005) (Figure 3B). Addition of dithiothreitol (DTT) reduces the disulfide bonds, and SecA can monomerize. The crosslinked SecA was tested in in vitro translocation assays using the fluorescently labeled fusion protein proOmpA-Dhfr-Atto550 and urea-treated IMVs of an *E. coli* strain containing overexpressed levels of SecYEG (Figure 3A). Translocated proOmpA-Dhfr-Atto550 is protected against an externally added protease and was visualized on SDS-PAGE by fluorescent imaging. Because absorption spectra of FM and Atto550 overlap, proOmpA-Dhfr-Atto550 and SecA-FM can be visualized simultaneously (Figure 3A). Crosslinked SecA migrates at around 200 kDa, and this state was maintained during the translocation-reaction conditions (Figure 3B). The efficiency of proOmpA-Dhfr-Atto550 translocation was nearly identical for the crosslinked and non-crosslinked SecA (Figure 3A), indicating that all subunits of the crosslinked SecA dimer are active. Importantly, under the conditions tested, SecA is the limiting factor, which is evident by a linear increase of the translocation efficiency with the SecA concentration (Figure 3A, compare lanes 2–4 and 5–6). These observations demonstrate that the fluorescently labeled SecA is active for protein translocation and that SecA is dimeric during protein translocation, at least during a rate-limiting step. However, these experiments cannot exclude that SecA monomerizes at a later step of protein translocation that is not rate limiting.

SecA Binds SecYEG as a Dimer throughout Protein Translocation

To further determine the oligomeric state of SecA when bound to SecYEG, the binding of the covalently crosslinked FM-labeled SecA dimer was compared with the non-crosslinked SecA at low nanomolar concentrations. The binding curves of

crosslinked and non-crosslinked SecA obtained by DCFBA are virtually identical, suggesting that the non-crosslinked SecA also binds as a dimer. Both curves saturate at a SecA/SecYEG ratio of approximately 0.3 when the linear nonsaturable binding parameter is subtracted from the fit (Figure 2A). No significant difference in nonspecific lipid binding of the crosslinked and non-crosslinked SecA-FM was observed, as assayed with *E. coli* liposomes labeled with the fluorescent lipid analog DiD (Figure 2B). The data strongly suggest that the non-crosslinked SecA binds SecYEG as a dimer. To determine the oligomeric state of translocation-engaged SecA, the binding reactions were supplemented with the preprotein proOmpA with (see Figure S1 available online) and without ATP (Figure S2) or with ADP (Figure S3). In all cases the binding curves of the crosslinked and non-crosslinked SecA dimer were identical and saturated at a ratio of about 0.3. The dissociation constants of SecA binding to SecYEG in absence or presence of translocation ligands and nucleotides were derived from the DCFBA-binding curves. Interestingly, the sensitive DCFBA technique allowed the detection of a 2.8-fold increase in the affinity of SecA to SecYEG in the presence of proOmpA (Figure 2C; 1.39 ± 0.22 nM and 1.28 ± 0.38 nM for crosslinked and non-crosslinked SecA, respectively, which compares to a value of 3.6 nM in the absence of proOmpA). Addition of proOmpA plus ATP leads to further affinity increase (0.87 ± 0.29 nM and 0.72 ± 0.08 nM for crosslinked and non-crosslinked SecA, respectively). On the other hand, addition of ADP resulted in a 2-fold decrease of the affinity of SecA to SecYEG compared to binding in the absence of translocation ligands and nucleotides (6.6 ± 1.8 nM and 7.02 ± 1.28 nM for crosslinked and non-crosslinked SecA, respectively).

SecA Binds SecYEG as a Monomer in the Presence of High Salt or Urea

We investigated the binding and oligomeric state of SecA at salt concentrations that were previously shown to favor dissociation of the SecA dimer in solution. In the presence of 500 mM NaCl, crosslinked SecA still binds SecYEG with high affinity (Figure 4), although with a somewhat reduced ratio (0.27) compared to the binding in absence of NaCl (0.32). In contrast the non-crosslinked SecA binds with a substantially reduced ratio to SecYEG (0.18, Figure 4) in the presence of high NaCl. The same 1.5-fold reduced ratio of non-crosslinked SecA binding to SecYEG was observed in the presence of 1 M urea (Figure S4). Considering the crosslinking efficiency of about 70% (Figure 3B), the reduction in the ratio suggests that in the presence of 500 mM NaCl or 1 M urea, non-crosslinked SecA binds mostly as a monomer. As anticipated, the unspecific binding of SecA to liposomes in the presence of 500 mM NaCl was significantly reduced, as assayed with empty *E. coli* liposomes labeled with the fluorescent lipid analog DiD (data not shown). Consequently, the linear parameter in the binding of SecA to SecYEG containing proteoliposomes was reduced.

Measuring oligomerization of soluble proteins with low (submicromolar) dissociation constants by standard methods is often limited by low detection sensitivity. Indeed, previous attempts to determine the dissociation constant for SecA dimerization were unsuccessful because of the limited sensitivity of sedimentation velocity centrifugation, yielding estimates in the range of 100 nM (Woodbury et al., 2002) to <0.6 μM (Doyle et al., 2000).

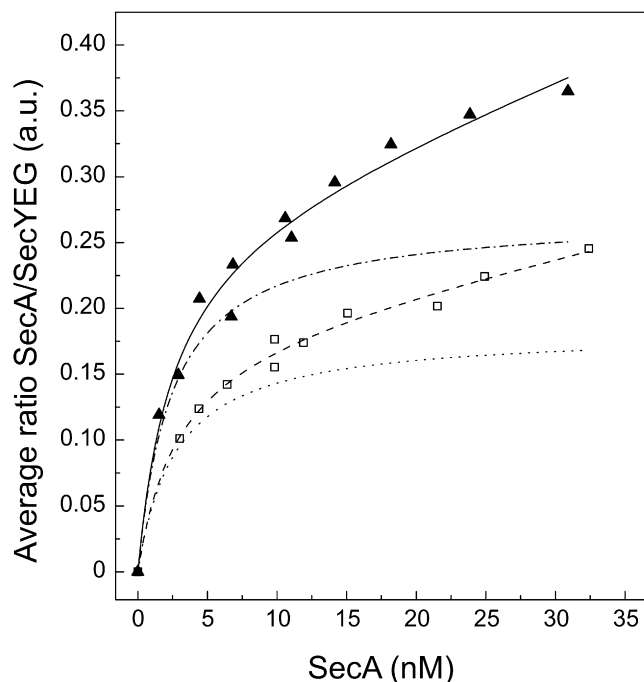


Figure 4. High Salt Sensitivity of Binding of the SecA dimer to SecYEG

Binding of crosslinked (triangles) and non-crosslinked SecA (squares) to SecYEG in the presence of 500 mM NaCl. Data points were fitted using the Hill equation (straight and dashed lines), and the linear nonsaturable binding parameter was subtracted from the fit (dotted lines).

Fluorescence cross-correlation spectroscopy (FCCS) enables the detection of heterodimers of protein subunits labeled with two spectrally distinct fluorophores at low nanomolar concentrations (Bacia and Schwille, 2007). Simultaneously, the concentration of all fluorescent species is determined. A dilution series of a mixture of SecA-Atto488 and SecA-Cy5 with a high degree of dual-color heterodimers was subjected to FCCS measurements to determine the dissociation constant for SecA dimerization. The binding curve resulting from the relative concentration of dual-color SecA heterodimers plotted against the SecA concentration revealed a K_d for the dissociation of the SecA dimer of 0.74 ± 0.09 nM (Figure 5A). This implies that both dimerization of SecA in solution and binding of dimeric SecA to SecYEG occur at similarly low nanomolar concentration, underlining the importance of dimeric SecA in the translocation cycle. To investigate the influence of NaCl on dimerization of SecA in solution, dual-color SecA (28 nM) was treated with various NaCl concentrations and measured by FCCS. Evidently, the relative concentration of dual-color SecA heterodimers decreased with increasing NaCl concentrations (Figure 5B). At 500 mM NaCl the vast majority of SecA was monomeric. This observation indicates that SecA only binds SecYEG as a monomer when previously monomerized in solution.

To assay the influence of high salt on the translocation activity of crosslinked and non-crosslinked SecA, we performed in vitro translocation experiments with varying NaCl concentrations. Inhibition of protein translocation was observed at >200 mM NaCl (Figure 6). Interestingly, the translocation

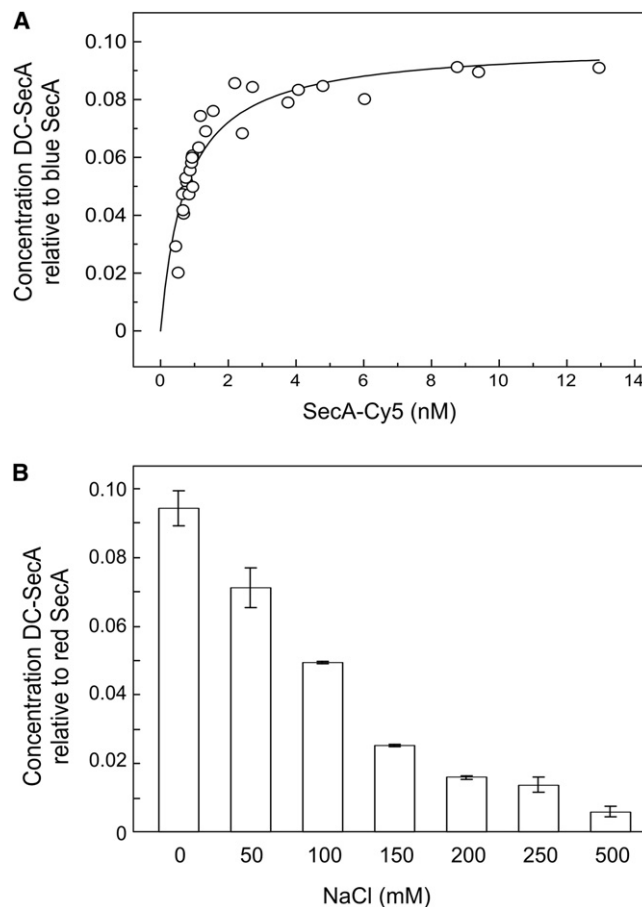


Figure 5. Dimerization of SecA in Solution

(A) Dimerization of SecA in suspension monitored by FCCS. The relative concentration of dual-color SecA heterodimers decreases when a mixture of SecA, labeled with two distinct fluorophores, is diluted to low nanomolar concentrations. The dissociation constant was calculated from the Hill fit. (B) Influence of NaCl on the dimerization of SecA in suspension monitored by FCCS. SecA (28 nM) was incubated with different NaCl concentrations, and the relative concentration of dual-color SecA heterodimers was determined by FCCS. Error bars show SD.

efficiency was affected significantly more with non-crosslinked SecA compared to crosslinked SecA (compare lanes 2–6 with 7–11 in Figure 6), demonstrating that stably dimeric SecA is more efficient in protein translocation at high salt concentrations.

Protein Translocation Requires Cycling of SecA

To examine the apparent Michaelis-Menten constant (K_M) of SecA for protein translocation, we performed in vitro translocation experiments with varying SecA concentrations under the same conditions as used in the DCFBA measurements. The translocation of fluorescently labeled proOmpA into proteoliposomes containing SecYEG saturated at high nanomolar concentration with an apparent K_M of 106 ± 16 nM (Figure 7). Compared to the high binding affinity of SecA to SecYEG, this relatively low apparent K_M demonstrates that an excess of SecA is necessary for efficient protein translocation.

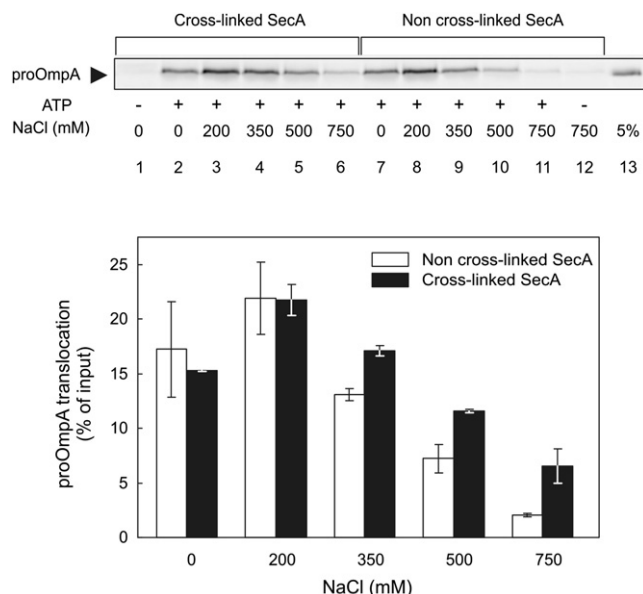


Figure 6. High Salt Sensitivity of Protein Translocation

In vitro protein translocation in the presence of NaCl. Crosslinked SecA (lanes 2–6) is less inhibited by increasing NaCl concentrations than non-crosslinked SecA (lanes 7–11). NaCl concentrations were 0, 200, 350, 500, and 750 mM for lanes 2–6 and 7–11, respectively. Reactions in lanes 1 and 12 were without ATP (digestion controls). Lane 13 shows 5% of input material. Graph represents quantified bands from two independent experiments. Error bars show the spread.

DISCUSSION

Measuring the quaternary structure and high-affinity binding of ligands to their membrane receptors under physiologically relevant conditions has been a challenge up to date. The low detection sensitivity for proteins as well as the handling of membrane proteins, detergent-solubilized or embedded in an elastic and sensitive membrane environment, have hampered such analysis. Here, we employ DCFBA to probe the quaternary structure of the motor protein SecA when bound to its membrane-embedded receptor, the protein-conducting channel SecYEG. Simultaneously, the method allowed the determination of the binding affinity of SecA to SecYEG for various steps of the translocation cycle.

In this study we show by employing an in vitro translocation assay that crosslinked (covalent dimer) and non-crosslinked (noncovalent dimer) SecA have identical activity, yet being the limiting factor. This result demonstrates that, at least during the rate-limiting step, SecA is dimeric in protein translocation. To investigate whether SecA monomerizes at a later, nonrate-limiting, step of the translocation cycle, we used fluorescently labeled SecA in the crosslinked and non-crosslinked form for a binding assay followed by DCFBA. The average ratio of SecA over SecYEG as determined from the binding curves was virtually identical for all translocation conditions (no translocation ligands, preprotein, preprotein plus ATP, ADP), which implies that non-crosslinked SecA also binds as a dimer (Figure 2; Figures S1–S3). Previous studies concluding that SecA functions as a dimer (de Keyser et al., 2005; Driessen, 1993; Jilaveanu and

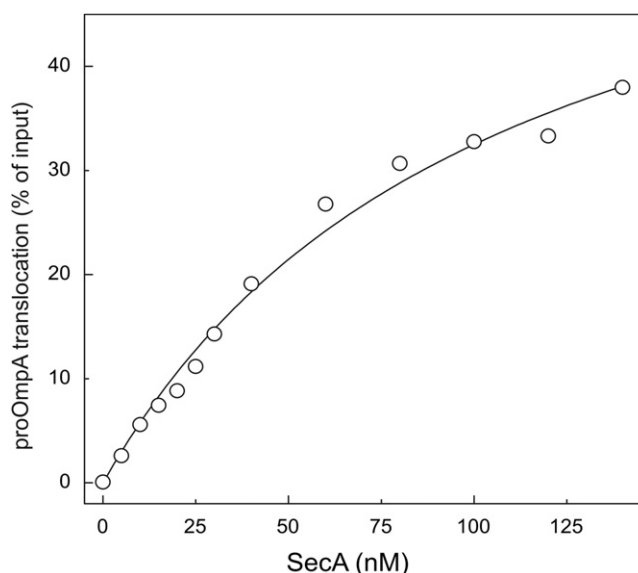


Figure 7. SecA Dependence of Protein Translocation

In vitro protein translocation of proOmpA-FM into SecYEG containing proteoliposomes as a function of the SecA concentration. Solid line presents a fit using the Michaelis-Menten equation.

Oliver, 2006) were recently opposed by a crystal structure of a monomeric SecA-SecYEG complex (Zimmer et al., 2008) from *Thermotoga maritima*. However, this structure was obtained in detergent and at high salt concentration, both conditions that favor dissociation of the SecA dimer (Benach et al., 2003; Or et al., 2002; Woodbury et al., 2002) (this study). Interestingly, the residues mediating dimerization of *E. coli* SecA (Papanikolaou et al., 2007) are highly conserved, and the corresponding residues of the *T. maritima* SecA are exposed to the cytoplasmic surface in the SecYEG-bound state (Zimmer et al., 2008) (compare Figures 8A and 8B). Therefore, it is possible that a second SecA protomer associates with these structurally conserved motifs of SecYEG-bound SecA. We visualized such SecA dimer by docking a duplicated copy of the *T. maritima* SecA protomer onto the SecA-SecYEG structure (Figure 8C) using the coordinates of the *E. coli* dimer (Figure 8D). It is likely that the second protomer has a different conformation as the SecYEG-bound protomer because nucleotide binding induces a dramatic structural change in SecA, e.g., rotation of the preprotein-binding domain (PBD) by around 80° (Zimmer et al., 2008). However, the cytoplasmic surface of SecYEG-bound SecA seems less affected by these changes and readily allowed docking of the second SecA protomer to the conserved dimer interface of the *E. coli* SecA. Interestingly, in this antiparallel dimer arrangement, the two PBDs come in close proximity, which would enable transfer of preprotein segments from one protomer to the other. However, it should be emphasized that the dimerization interfaces in the crystal structures of various SecA proteins differ substantially (Ding et al., 2003; Hunt et al., 2002; Papanikolaou et al., 2007; Sharma et al., 2003; Vassilyev et al., 2006; Weinkauff et al., 2001), and it remains unclear which dimeric conformation is physiological relevant (for review, see Sardis and Economou, 2010). Importantly, the orientation of

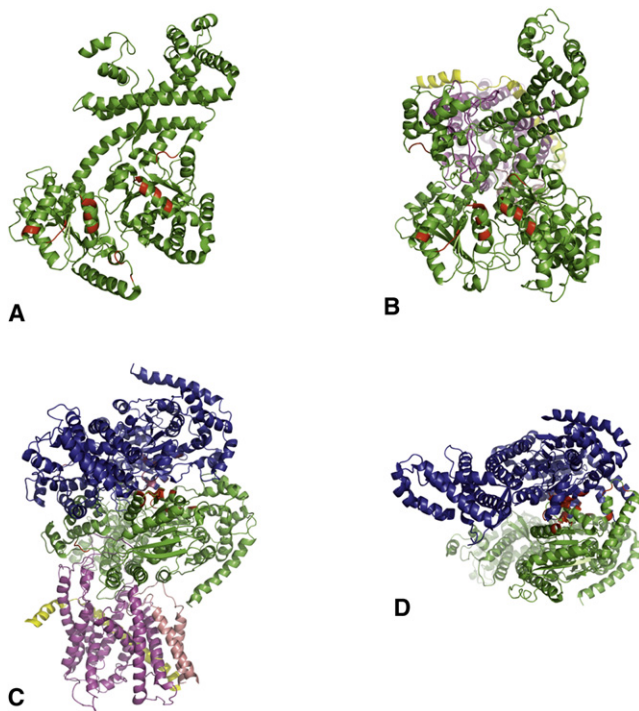


Figure 8. Structural Model for the Dimeric SecA Bound to SecYEG
Structures of the *E. coli* and *T. maritima* SecA and a structural model for the dimeric SecA bound to SecYEG. The dimer interface of *E. coli* SecA (A, residues in red) (Papanikolaou et al., 2007) (PDB accession code 2FSF) is highly conserved and (B) exposed on the cytoplasmic surface of SecYEG-bound SecA of *T. maritima* (Zimmer et al., 2008) (PDB accession code 3DIN). Docking of a second copy of the *T. maritima* SecA onto the monomeric SecYEG-bound protomer (C) using the coordinates of the *E. coli* SecA dimer (D). The SecA protomers are shown in green and blue, respectively. The *T. maritima* SecY, SecE, and SecG are shown in purple, yellow, and pink, respectively. Structural models were produced with PyMol (<http://pymol.sourceforge.net/>). See also Figure S5.

the SecA monomer in the SecA-SecYEG structure is not compatible with several of the proposed crystallographic SecA dimers and also contradicts some biochemical crosslinking studies aimed at elucidating the orientation of the SecA dimer. Our model also fits the crystal structure of the *B. subtilis* SecA dimer (Hunt et al., 2002). The corresponding residues in the HSD that are involved in dimerization in the *B. subtilis* SecA dimer are exposed to the cytoplasm in the *T. maritima* SecA-SecYEG cocrystal (Figures S5A and S5B). However, the flexible N terminus of SecA that has been implicated in dimerization of the *B. subtilis* SecA is oriented toward the membrane in the SecA-SecYEG structure. However, it should be noted that there is conflicting evidence regarding the importance of the N terminus. In one study (Or et al., 2005), an N-terminal deletion in the *E. coli* SecA led to monomerization and inactivation of SecA, whereas in other studies (Karamanou et al., 2005; Wang et al., 2008), a similar mutant was found to be dimeric and fully functional. Additionally, the genetically fused head-to-tail (N to C terminus) (Wang et al., 2008) or chemically crosslinked (C to C terminus) SecA dimers (Figure 3) (de Keyzer et al., 2005) are fully active in protein translocation. Thus, the involvement of

the extreme termini in physiological dimerization remains obscure. The *E. coli* SecA dimer has also been crosslinked via introduced cysteines at positions 11/611 and 637/801 (Jilaveanu and Oliver, 2006) while retaining some activity and at 636/801 (Or and Rapoport, 2007), resulting in inactivation of the protein. The residues 611, 636, 637, and 801 are localized at the binding interface of the *T. maritima* SecA-SecYEG and, thus, cannot participate both in SecYEG binding and SecA dimerization (Figure S5C). Hence, both the structure and our model are compatible with this crosslinking data. Similarly, the residues implicated in dimerization of the antiparallel dimer crystallized from *M. tuberculosis* are close or within the SecA-SecYEG binding interface and, thus, cannot participate in dimerization, as depicted in Figure 8. Also, the short helix that participates in the formation of the parallel *T. thermophilus* SecA dimer is oriented sideways in the *T. maritima* SecA-SecYEG structure (Figures S5A and S5B). Finally, the ringlike structures of membrane-bound SecA observed by EM are difficult to conceive with our model. Importantly, our model of SecA dimerization is compatible with the *T. maritima* SecA-SecYEG cocrystal, the *E. coli* and *B. subtilis* SecA dimer structures, and is supported by various C- and N-terminal crosslink studies. Furthermore, it combines previous conflicting functional studies that reported binding of monomeric and/or dimeric SecA to SecYEG (de Keyzer et al., 2005; Duong, 2003; Jilaveanu et al., 2005; Or et al., 2002; Tziatzios et al., 2004). The interaction between SecA and SecYEG may involve only one protomer of the SecA dimer, whereas the second copy does not interact with SecYEG directly, as shown in Figure 8C. Therefore, association of monomeric SecA is also possible, and the relevant quaternary structure of the SecA-SecYEG complex is determined by the sensitive nature of the SecA monomer-dimer equilibrium. We now show that binding of monomeric SecA to SecYEG is salt resistant, whereas binding of the second protomer is salt sensitive (Figure 4). In earlier studies on the oligomeric state of the SecYEG-bound SecA, the experimental conditions varied considerably. The use of detergents (Duong, 2003; Tziatzios et al., 2004), low protein concentration (Or et al., 2002), or nonequilibrium methods (gel filtration, native gel electrophoresis) may have shifted the monomer-dimer equilibrium of SecA toward the monomeric form. It is well established that an increasing salt concentration leads to monomerization of SecA in solution (Woodbury et al., 2002). Therefore, to probe the binding of monomeric SecA to membrane-embedded SecYEG, we performed the DCFBA binding assay in the presence of 500 mM NaCl (or 1 M urea). For all conditions, non-crosslinked SecA bound with significantly lower ratio to SecYEG than crosslinked SecA, indicating salt-resistant monomeric SecA binding. Considering a crosslinking efficiency of about 70%, the 1.5-fold lower ratio of non-crosslinked SecA binding to SecYEG at 500 mM NaCl implies that the majority of SecA is bound as a monomer. We performed FCCS experiments with SecA labeled with two spectrally distinct fluorophores confirming that increasing salt concentrations shift the monomer-dimer equilibrium of SecA toward the monomeric state (Figure 5B). The salt sensitivity suggests that electrostatic interactions play a critical role in dimerization. Indeed, the *E. coli* SecA dimer is stabilized by 15 hydrogen bonds, although the primary dimer interface involves hydrophobic contacts (Papanikolaou et al., 2007). Remarkably,

the affinity (K_d of 0.74 nM) for dimerization (in solution) is significantly higher than the affinity of SecA for SecYEG. This implies that at SecYEG saturating concentrations, SecA will bind as a dimer. Our quantitative assessment of the dissociation constants indicates that the monomer-dimer equilibrium of SecA in solution determines the oligomeric state of the SecYEG-bound SecA and that SecYEG does not induce SecA monomerization.

DCFBA is based on the quantification of fluorescent bursts that reside from fluorescently labeled proteins diffusing through the two laser foci of a dual-color confocal microscope (Figure 1A). Co-migrating proteins, labeled with two spectrally distinct fluorophores, will result in fluorescent bursts whose intensities depend on the position within the confocal excitation volume and the number of fluorophores in the complex (Figure 1B). Thus, changes in the relative stoichiometry of the co-migrating proteins can be addressed by quantifying the intensity of overlapping fluorescent bursts and calculating the relative ratio of one color over the other. In comparison with similar methods that use coincidence or burst selection, such as confocal fluorescence coincidence analysis (CFCA) (Heinze et al., 2002; Winkler et al., 1999; Yao et al., 2005) or two-color coincidence detection (TCCD) (Green et al., 2006; James et al., 2007; Li et al., 2003), DCFBA offers several advantages. Two arbitrary offsets are used to select the overlapping bursts, and not only is the fraction of coincidence revealed, but the relative concentration of co-migrating fluorophores is determined with single-particle resolution. This is particularly important when complexes with a variable number of fluorophores are present, as is the case using liposomes with heterogeneous size distribution and membrane protein content. However, DCFBA underlies a single-particle analysis, and at higher (>20 nM) nanomolar concentration, on average more than one fluorescent species resides in the focal volume, leading to an increasing degree of coincidental co-migration with increasing particle number. Therefore, a concentration-dependent linear increase in co-migrating fluorescent particles is observed at high nanomolar concentrations.

The activity of monomeric wild-type SecA, as assayed by in vitro translocation, in the presence of high salt is significantly more inhibited than that of the dimeric, crosslinked, SecA (Figure 6). This is in agreement with earlier studies where SecA mutants with the monomer-dimer equilibrium shifted toward the monomeric state had low or no activity at all (Jilaveanu et al., 2005; Or et al., 2002, 2005; Randall et al., 2005). The increase of translocation activity in the presence of 200 mM NaCl is most likely due to a significant reduction in proteoliposome aggregation, which occurs under in vitro translocation assay conditions as observed by fluorescence microscopy (data not shown). In line with the above observations, in previous studies heterodimers composed of active and nonactive SecA monomers were shown to be entirely inactive (Driessen, 1993). Overall, our data now show that SecA is dimeric throughout the protein translocation cycle and that monomeric SecA has a low or no translocation activity.

Does a single SecA dimer bound to SecYEG mediate the translocation of an entire preprotein, or are repeated cycles of SecA dimer binding and dissociation from SecYEG required for complete translocation? Our data on the in vitro translocation

assay with varying SecA concentrations and the affinity cascade of SecA for SecYEG in the presence of translocation ligands provide evidence for a cycling model. Remarkably, the affinity of SecA to SecYEG increases significantly in the presence of preprotein and by the addition of ATP. In contrast, in the presence of ADP, there is a reduction in the binding affinity, confirming previous reports (van der Wolk et al., 1998). The apparent K_M derived from the in vitro translocation assay with varying SecA concentrations (106 nM, Figure 7) is about 30-fold higher than the K_d for SecA binding to SecYEG (3.6 nM, Figure 2) and even more than 150-fold higher than the K_d for the dissociation of the SecA dimer (0.74 nM, Figure 5A). The large difference between the K_d and apparent K_M indicates that a single SecA bound to SecYEG is highly inefficient in protein translocation in vitro and suggests that cycles of SecA binding and complete dissociation from the SecYEG channel are required for protein translocation. Our observations support the notion that protein translocation is driven by repeated nucleotide binding and hydrolysis-driven cycles of SecA binding and dissociation (Schiebel et al., 1991; van der Wolk et al., 1997). Future studies should address the question how the two protomers contribute to the protein translocation reaction during the catalytic cycle of SecA.

EXPERIMENTAL PROCEDURES

Chemical and Reagents

FM and DiD were purchased from Molecular Probes (Invitrogen), maleimide derivatives of Atto647N, Atto550, and Atto488 from Atto-Tec (57076 Siegen, Germany), and Cy5-maleimide from GE-Healthcare. Cation exchange chromatography was performed on HiTrap SP HP and gel filtration on Superdex 200 XK26/60 or NAP5 columns from GE Healthcare. HIS-Select Nickel Affinity Gel (P6611) was purchased from Sigma-Aldrich. N-Dodecyl- β -maltoside (DDM) was purchased from Anatrace. *E. coli* total lipid extract was purchased from Avanti and acetone-ether washed as described elsewhere (van der Does et al., 2003). Bio-Beads SM-2 adsorbents, Micro Bio-Spin 6, and empty Bio-Spin chromatography columns were purchased from Bio-Rad.

Protein Purification and Reconstitution

SecB and proOmpA were purified as described elsewhere (Kusters et al., 2010). SecA was purified via a single-step cation exchange chromatography. Cell-free extract (in 20 mM HEPES-KOH [pH 6.5]) of *E. coli* DH5 α carrying plasmid pMKLI8 (van der Does et al., 2003) grown overnight in LB to OD 0.6 was applied on a HiTrap SP HP column equilibrated with buffer A (20 mM HEPES-KOH [pH 6.5], 10% glycerol). The column was washed with buffer A supplemented with 100 mM NaCl, and elution was achieved by a NaCl gradient in buffer A. SecA eluted around 400 mM NaCl. Prior to labeling with a 20-fold molar excess of FM at pH 7, SecA was incubated for 30 min with a 10-fold molar excess of ZnCl₂. The labeling reaction was stopped after 3 hr by addition of 1 mM DTT. In order to remove the Zn²⁺, a 10-fold molar excess of EDTA was added, followed by 30-min incubation. Prior to the labeling of WT-SecA with Atto488 and Cy5, Zn²⁺ ions were chelated by incubation for 30 min with a 10-fold molar excess of EDTA. Fluorescently labeled SecA was then purified on a Superose 12 column with Buffer B (50 mM Tris-HCl [pH 7.6], 10% [v/v] glycerol, 50 mM KCl). Crosslinking of SecA was achieved by incubation with 0.5 mM CuPhe for 30 min at room temperature (RT). To remove CuPhe, SecA was applied on a Micro Bio-Spin 6 gel filtration column equilibrated with Buffer B. SecY_(C295)EG purification, labeling with Atto647N-maleimide, and reconstitution into total *E. coli* lipid extract are described elsewhere (Kusters et al., 2010). The labeling efficiency of both SecA-FM and SecY_(C295)EG-Atto647N was determined by UV-Vis spectroscopy and corrected for the fluorophore absorption at 280 nm, as specified by the manufacturer of the fluorophores. Labeling efficiency was 100% for SecA-FM and approximately 80% for SecY_(C295)EG-Atto647N.

In Vitro Protein Translocation

The in vitro protein translocation assays were essentially performed as described elsewhere (Kusters et al., 2010) with the following variations. For assaying crosslinked SecA-FM the translocation mixture contained urea-treated IMVs of *E. coli* SF100 overexpressing SecY_(C295)EG, and 2 mM ATP. Translocation assays were performed in the absence of SecB. Indicated concentrations of crosslinked or non-crosslinked SecA were added to the mixture, and the volume was corrected with buffer B. After 3-min incubation at 37°C, the reaction was started by the addition of 20 µg/ml proOmpA-Dhfr-Atto550 (unfolded Dhfr domain). For in vitro translocation in the presence of NaCl at RT, a translocation mixture with SecY_(C295)EG containing proteoliposomes, 100 nM crosslinked or non-crosslinked SecA, and 20 µg/ml proOmpA-FM was adjusted to the indicated NaCl concentration and incubated for 10 min at RT before the reaction was started by addition of 2 mM ATP and continued for 45 min at RT. The translocation mixture for K_M determination was composed of SecY_(C295)EG proteoliposomes, 80 µg/ml SecB, 20 µg/ml proOmpA-FM, indicated SecA concentrations, and carried out at RT as described above.

Binding Reactions and Sample Preparation

Binding of SecA-FM to SecY_(C295)EG-Atto647N reconstituted into *E. coli* liposomes was performed as follows. SecA-FM (2–80 nM) was incubated with proteoliposomes containing SecY_(C295)EG-Atto647N in buffer C (50 mM HEPES-KOH [pH 7.6], 30 mM KCl, 2 mM MgCl₂) for 12 min at RT. For the DCFBA measurements a microscope sample of 10 µl was prepared with a silicone Press-to-Seal spacer (Invitrogen). For the FCCS experiments, equimolar amounts of SecA-Atto488 and SecA-Cy5 were mixed and incubated with 500 mM NaCl for 30 min on ice to dissociate SecA dimers. Buffer exchange to buffer C and formation of SecA-heterodimers were achieved by gel filtration (NAP5 column; GE Healthcare). A 30-min ultracentrifugation at 400,000 × g ensured the absence of aggregates in the sample.

Fluorescence Spectroscopy, DCFBA, and FCCS Analysis

All fluorescence measurements were carried out on a dual-color LSCM. The LSCM was based on an inverted Axiovert S 100 TV microscope (Zeiss) and combined with a galvanometer optical scanner (model 6860; Cambridge Technology). The two laser beams (488 nm, argon ion laser, Spectra-Physics; 633 nm, He-Ne laser, JDS Uniphase) were focused by a Zeiss C-Apochromat infinity-corrected 1.2 NA 40× water-immersion objective. The fluorescence was collected through the same objective, separated from the excitation beams by a beam pick-off plate (BSP20-A1, Thor-Labs), split into two channels by a dichroic beam splitter (585DCXR; Chroma Technology), and finally directed through emission filters (HQ 535/50 and HQ 675/50; Chroma Technology) and pinholes (diameter of 30 µm) onto two avalanche photodiodes (SPCM-AQR-14; EG&G). For the laser-scanning DCFBA (lsDCFBA) (for review see van den Bogaart et al., 2008) measurements, the two aligned laser beams were moved simultaneously with the galvanometer optical scanner through the sample well above the surfaces of the sample holder. Two confocal images (60 × 60 µm) of SecA-FM and SecY_(C295)EG-Atto647N containing proteoliposomes were recorded simultaneously. The fluorescent bursts originating from the proteoliposomes and SecA-FM were identified using arbitrary fluorescence offsets as described (van den Bogaart et al., 2008). Software for DCFBA can be downloaded at <http://www.bogeert.com/DCFBA/publish.htm>. The dwell time of the laser scanning was 200 µs, and the pixel step was 110 nm. Typically, in four consecutive scans, 100–1000 bursts were recorded for sufficient statistics. A dilution series of SecA-FM was subjected to FCS measurements and confocal scans. The concentration of SecA-FM was calculated from the amplitude of the FCS curve and plotted against the total fluorescence from the corresponding scan. The resulting calibration curve was used to determine the concentration of SecA-FM in the scans from binding assays. The FCCS and data analysis were performed as described elsewhere (Veldhuis et al., 2006).

For the analysis a number of controls were performed to ascertain that quenching does not occur during the measurement. The fluorescent intensity (counts per molecule, cpm) of crosslinked and non-crosslinked SecA as determined by FCS showed identical cpm. Furthermore, the total fluorescence as derived from the confocal scans was comparable for both species at similar protein concentration. The Atto647N fluorophore that is insensitive to

environmental changes was attached to a periplasmic loop of SecY, keeping it far away from the cytoplasmic SecA-binding site. This, besides the spectral separation of fluorescein and Atto647N, further diminishes the chance for Förster Resonance Energy Transfer (FRET) between fluorophores on SecA and SecY. Finally, background fluorescence originating from fluorescent molecules diffusing far from the center of the confocal excitation volume and dark current was filtered out by the arbitrary offsets as described (van den Bogaart et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.str.2010.12.016.

ACKNOWLEDGMENTS

This work was supported by NanoNed, a national nanotechnology program coordinated by the Dutch Ministry of Economical Affairs and the Zernike Institute for Advanced Materials, and by the Chemical Sciences division of The Netherlands Organization for Scientific Research (NWO-CW).

Received: September 3, 2010

Revised: December 17, 2010

Accepted: December 22, 2010

Published: March 8, 2011

REFERENCES

- Akita, M., Shinkai, A., Matsuyama, S., and Mizushima, S. (1991). SecA, an essential component of the secretory machinery of *Escherichia coli*, exists as homodimer. *Biochem. Biophys. Res. Commun.* 174, 211–216.
- Bacia, K., and Schwille, P. (2007). Practical guidelines for dual-color fluorescence cross-correlation spectroscopy. *Nat. Protoc.* 2, 2842–2856.
- Benach, J., Chou, Y.T., Fak, J.J., Itkin, A., Nicolae, D.D., Smith, P.C., Wittrock, G., Floyd, D.L., Golsaz, C.M., Gierasch, L.M., and Hunt, J.F. (2003). Phospholipid-induced monomerization and signal-peptide-induced oligomerization of SecA. *J. Biol. Chem.* 278, 3628–3638.
- Bessonneau, P., Besson, V., Collinson, I., and Duong, F. (2002). The SecYEG preprotein translocation channel is a conformationally dynamic and dimeric structure. *EMBO J.* 21, 995–1003.
- Bu, Z., Wang, L., and Kendall, D.A. (2003). Nucleotide binding induces changes in the oligomeric state and conformation of Sec A in a lipid environment: a small-angle neutron-scattering study. *J. Mol. Biol.* 332, 23–30.
- de Keyzer, J., van der Does, C., Kloosterman, T.G., and Driessen, A.J. (2003). Direct demonstration of ATP-dependent release of SecA from a translocating preprotein by surface plasmon resonance. *J. Biol. Chem.* 278, 29581–29586.
- de Keyzer, J., van der Sluis, E.O., Spelbrink, R.E., Nijstad, N., de Kruijff, B., Nouwen, N., van der Does, C., and Driessen, A.J. (2005). Covalently dimerized SecA is functional in protein translocation. *J. Biol. Chem.* 280, 35255–35260.
- Ding, H., Mukerji, I., and Oliver, D. (2003). Nucleotide and phospholipid-dependent control of PPXD and C-domain association for SecA ATPase. *Biochemistry* 42, 13468–13475.
- Doyle, S.M., Braswell, E.H., and Teschke, C.M. (2000). SecA folds via a dimeric intermediate. *Biochemistry* 39, 11667–11676.
- Driessen, A.J. (1993). SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. *Biochemistry* 32, 13190–13197.
- Driessen, A.J., and Nouwen, N. (2008). Protein translocation across the bacterial cytoplasmic membrane. *Annu. Rev. Biochem.* 77, 643–667.
- Duong, F. (2003). Binding, activation and dissociation of the dimeric SecA ATPase at the dimeric SecYEG translocase. *EMBO J.* 22, 4375–4384.
- Economou, A., and Wickner, W. (1994). SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* 78, 835–843.

- Fekkes, P., de Wit, J.G., van der Wolk, J.P., Kimsey, H.H., Kumamoto, C.A., and Driessen, A.J. (1998). Preprotein transfer to the *Escherichia coli* translocase requires the co-operative binding of SecB and the signal sequence to SecA. *Mol. Microbiol.* 29, 1179–1190.
- Green, J.J., Ladame, S., Ying, L., Klennerman, D., and Balasubramanian, S. (2006). Investigating a quadruplex-ligand interaction by unfolding kinetics. *J. Am. Chem. Soc.* 128, 9809–9812.
- Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P., and Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell* 63, 269–279.
- Heinze, K.G., Rarbach, M., Jahnz, M., and Schwille, P. (2002). Two-photon fluorescence coincidence analysis: rapid measurements of enzyme kinetics. *Biophys. J.* 83, 1671–1681.
- Hunt, J.F., Weinkauff, S., Henry, L., Fak, J.J., McNicholas, P., Oliver, D.B., and Deisenhofer, J. (2002). Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. *Science* 297, 2018–2026.
- James, J.R., White, S.S., Clarke, R.W., Johansen, A.M., Dunne, P.D., Sleep, D.L., Fitzgerald, W.J., Davis, S.J., and Klennerman, D. (2007). Single-molecule level analysis of the subunit composition of the T cell receptor on live T cells. *Proc. Natl. Acad. Sci. USA* 104, 17662–17667.
- Jilaveanu, L.B., and Oliver, D. (2006). SecA dimer cross-linked at its subunit interface is functional for protein translocation. *J. Bacteriol.* 188, 335–338.
- Jilaveanu, L.B., Zito, C.R., and Oliver, D. (2005). Dimeric SecA is essential for protein translocation. *Proc. Natl. Acad. Sci. USA* 102, 7511–7516.
- Karamanou, S., Sianidis, G., Gouridis, G., Pozidis, C., Papanikolaou, Y., Papanikou, E., and Economou, A. (2005). *Escherichia coli* SecA truncated at its termini is functional and dimeric. *FEBS Lett.* 579, 1267–1271.
- Kusters, I., van den Bogaart, G., de Wit, J., Krasnikov, V., Poolman, B., and Driessen, A. (2010). Purification and functional reconstitution of the bacterial protein translocation pore, the SecYEG complex. *Methods Mol. Biol.* 619, 131–143.
- Li, H., Ying, L., Green, J.J., Balasubramanian, S., and Klennerman, D. (2003). Ultrasensitive coincidence fluorescence detection of single DNA molecules. *Anal. Chem.* 75, 1664–1670.
- Manting, E.H., van Der Does, C., Remigy, H., Engel, A., and Driessen, A.J. (2000). SecYEG assembles into a tetramer to form the active protein translocation channel. *EMBO J.* 19, 852–861.
- Meyer, T.H., Menetret, J.F., Breitling, R., Miller, K.R., Akey, C.W., and Rapoport, T.A. (1999). The bacterial SecY/E translocation complex forms channel-like structures similar to those of the eukaryotic Sec61p complex. *J. Mol. Biol.* 285, 1789–1800.
- Mori, H., Tsukazaki, T., Masui, R., Kuramitsu, S., Yokoyama, S., Johnson, A.E., Kimura, Y., Akiyama, Y., and Ito, K. (2003). Fluorescence resonance energy transfer analysis of protein translocase. SecYE from *Thermus thermophilus* HB8 forms a constitutive oligomer in membranes. *J. Biol. Chem.* 278, 14257–14264.
- Musial-Siwiek, M., Rusch, S.L., and Kendall, D.A. (2005). Probing the affinity of SecA for signal peptide in different environments. *Biochemistry* 44, 13987–13996.
- Or, E., and Rapoport, T. (2007). Cross-linked SecA dimers are not functional in protein translocation. *FEBS Lett.* 581, 2616–2620.
- Or, E., Navon, A., and Rapoport, T. (2002). Dissociation of the dimeric SecA ATPase during protein translocation across the bacterial membrane. *EMBO J.* 21, 4470–4479.
- Or, E., Boyd, D., Gon, S., Beckwith, J., and Rapoport, T. (2005). The bacterial ATPase SecA functions as a monomer in protein translocation. *J. Biol. Chem.* 280, 9097–9105.
- Papanikolaou, Y., Papadovasilaki, M., Ravelli, R.B., McCarthy, A.A., Cusack, S., Economou, A., and Petratos, K. (2007). Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. *J. Mol. Biol.* 366, 1545–1557.
- Randall, L.L., Crane, J.M., Lilly, A.A., Liu, G., Mao, C., Patel, C.N., and Hardy, S.J. (2005). Asymmetric binding between SecA and SecB two symmetric proteins: implications for function in export. *J. Mol. Biol.* 348, 479–489.
- Sardis, M.F., and Economou, A. (2010). SecA: a tale of two protomers. *Mol. Microbiol.* 76, 1070–1081.
- Schiebel, E., Driessen, A.J., Hartl, F.U., and Wickner, W. (1991). Delta mu H⁺ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell* 64, 927–939.
- Sharma, V., Arockiasamy, A., Ronning, D.R., Savva, C.G., Holzenburg, A., Braunstein, M., Jacobs, W.R., Jr., and Sacchettini, J.C. (2003). Crystal structure of *Mycobacterium tuberculosis* SecA, a preprotein translocating ATPase. *Proc. Natl. Acad. Sci. USA* 100, 2243–2248.
- Tziatzios, C., Schubert, D., Lotz, M., Gundogan, D., Betz, H., Schagger, H., Haase, W., Duong, F., and Collinson, I. (2004). The bacterial protein-translocation complex: SecYEG dimers associate with one or two SecA molecules. *J. Mol. Biol.* 340, 513–524.
- van den Bogaart, G., Krasnikov, V., and Poolman, B. (2007a). Dual-color fluorescence-burst analysis to probe protein efflux through the mechanosensitive channel MscL. *Biophys. J.* 92, 1233–1240.
- van den Bogaart, G., Mika, J.T., Krasnikov, V., and Poolman, B. (2007b). The lipid dependence of melittin action investigated by dual-color fluorescence burst analysis. *Biophys. J.* 93, 154–163.
- van den Bogaart, G., Kusters, I., Velasquez, J., Mika, J.T., Krasnikov, V., Driessen, A.J., and Poolman, B. (2008). Dual-color fluorescence-burst analysis to study pore formation and protein-protein interactions. *Methods* 46, 123–130.
- van der Does, C., de Keyser, J., van der Laan, M., and Driessen, A.J. (2003). Reconstitution of purified bacterial preprotein translocase in liposomes. *Methods Enzymol.* 372, 86–98.
- van der Sluis, E.O., Nouwen, N., and Driessen, A.J. (2002). SecY-SecY and SecY-SecG contacts revealed by site-specific crosslinking. *FEBS Lett.* 527, 159–165.
- van der Wolk, J.P., de Wit, J.G., and Driessen, A.J. (1997). The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events. *EMBO J.* 16, 7297–7304.
- van der Wolk, J.P., Fekkes, P., Boersma, A., Huie, J.L., Silhavy, T.J., and Driessen, A.J. (1998). PrlA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA-SecY interaction during the initiation of translocation. *EMBO J.* 17, 3631–3639.
- Vassilyev, D.G., Mori, H., Vassilyeva, M.N., Tsukazaki, T., Kimura, Y., Tahirov, T.H., and Ito, K. (2006). Crystal structure of the translocation ATPase SecA from *Thermus thermophilus* reveals a parallel, head-to-head dimer. *J. Mol. Biol.* 364, 248–258.
- Veenendaal, A.K., van der Does, C., and Driessen, A.J. (2001). Mapping the sites of interaction between SecY and SecE by cysteine scanning mutagenesis. *J. Biol. Chem.* 276, 32559–32566.
- Veldhuis, G., Hink, M., Krasnikov, V., van den Bogaart, G., Hoeboer, J., Visser, A.J., Broos, J., and Poolman, B. (2006). The oligomeric state and stability of the mannitol transporter, Enzymell(mtl), from *Escherichia coli*: a fluorescence correlation spectroscopy study. *Protein Sci.* 15, 1977–1986.
- Wang, H., Na, B., Yang, H., and Tai, P.C. (2008). Additional in vitro and in vivo evidence for SecA functioning as dimers in the membrane: dissociation into monomers is not essential for protein translocation in *Escherichia coli*. *J. Bacteriol.* 190, 1413–1418.
- Weinkauff, S., Hunt, J.F., Scheuring, J., Henry, L., Fak, J., Oliver, D.B., and Deisenhofer, J. (2001). Conformational stabilization and crystallization of the SecA translocation ATPase from *Bacillus subtilis*. *Acta Crystallogr. D Biol. Crystallogr.* 57, 559–565.
- Winkler, T., Kettling, U., Koltermann, A., and Eigen, M. (1999). Confocal fluorescence coincidence analysis: an approach to ultra high-throughput screening. *Proc. Natl. Acad. Sci. USA* 96, 1375–1378.
- Woodbury, R.L., Hardy, S.J., and Randall, L.L. (2002). Complex behavior in solution of homodimeric SecA. *Protein Sci.* 11, 875–882.
- Yao, J., Larson, D.R., Vishwasrao, H.D., Zipfel, W.R., and Webb, W.W. (2005). Blinking and nonradiant dark fraction of water-soluble quantum dots in aqueous solution. *Proc. Natl. Acad. Sci. USA* 102, 14284–14289.
- Zimmer, J., Nam, Y., and Rapoport, T.A. (2008). Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* 455, 936–943.

Supplemental Information

Quaternary Structure of SecA in Solution

and Bound to SecYEG Probed

at the Single Molecule Level

Ilja Kusters, Geert van den Bogaart, Alexej Kedrov, Victor Krasnikov, Faizah Fulyani, Bert Poolman, and Arnold J.M. Driessen

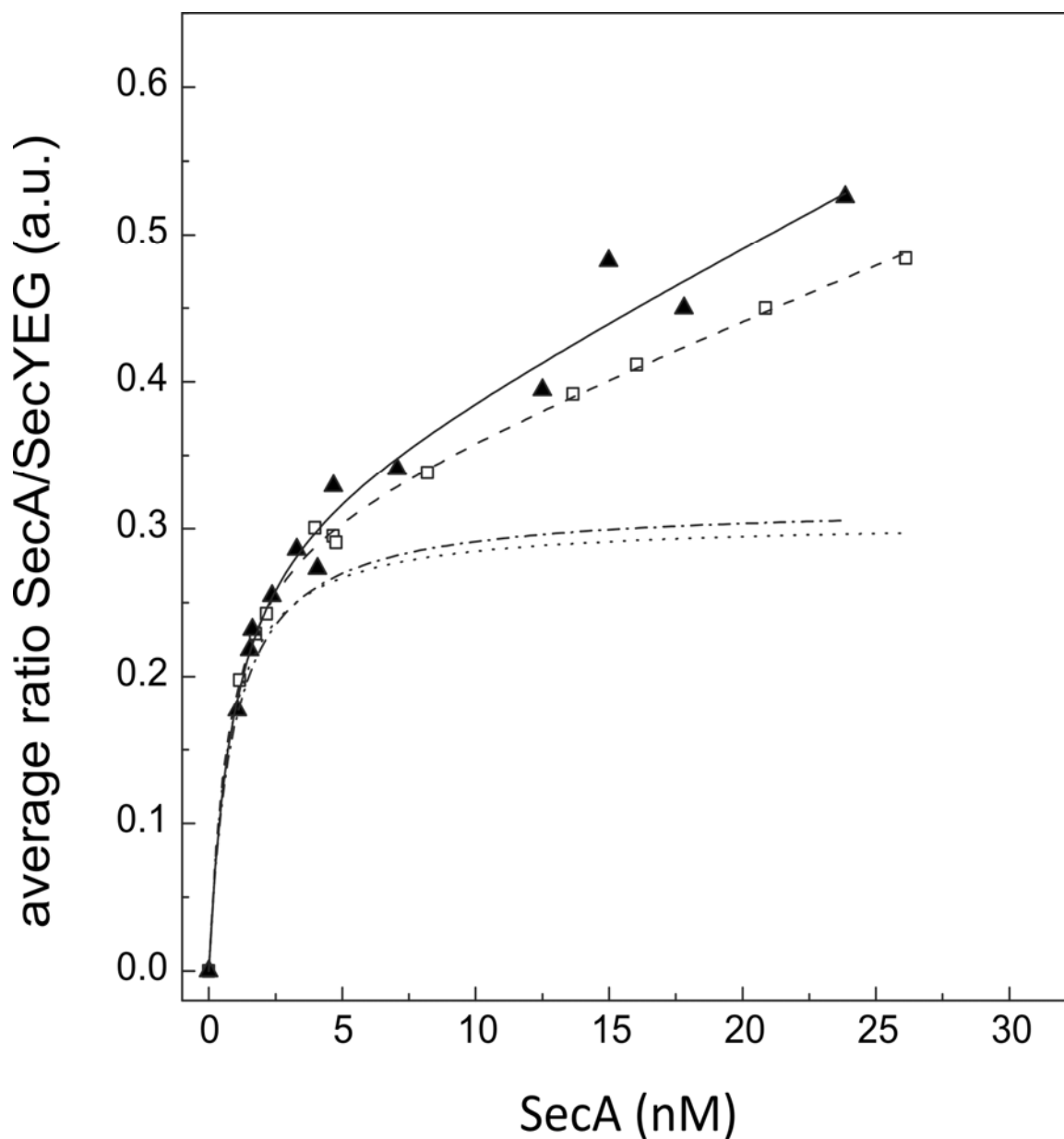


Figure S1, relates to Figure 2. Effect of proOmpA and ATP on SecA binding to SecYEG

Binding of cross-linked SecA-FM (triangles) and non cross-linked SecA-FM (squares) to SecYEG-Atto647N containing proteoliposomes in presence of proOmpA and 1mM ATP. Data points were fitted using the Hill-equation (straight and dashed lines) and the linear nonsaturable binding parameter was subtracted from the fit (dotted lines).

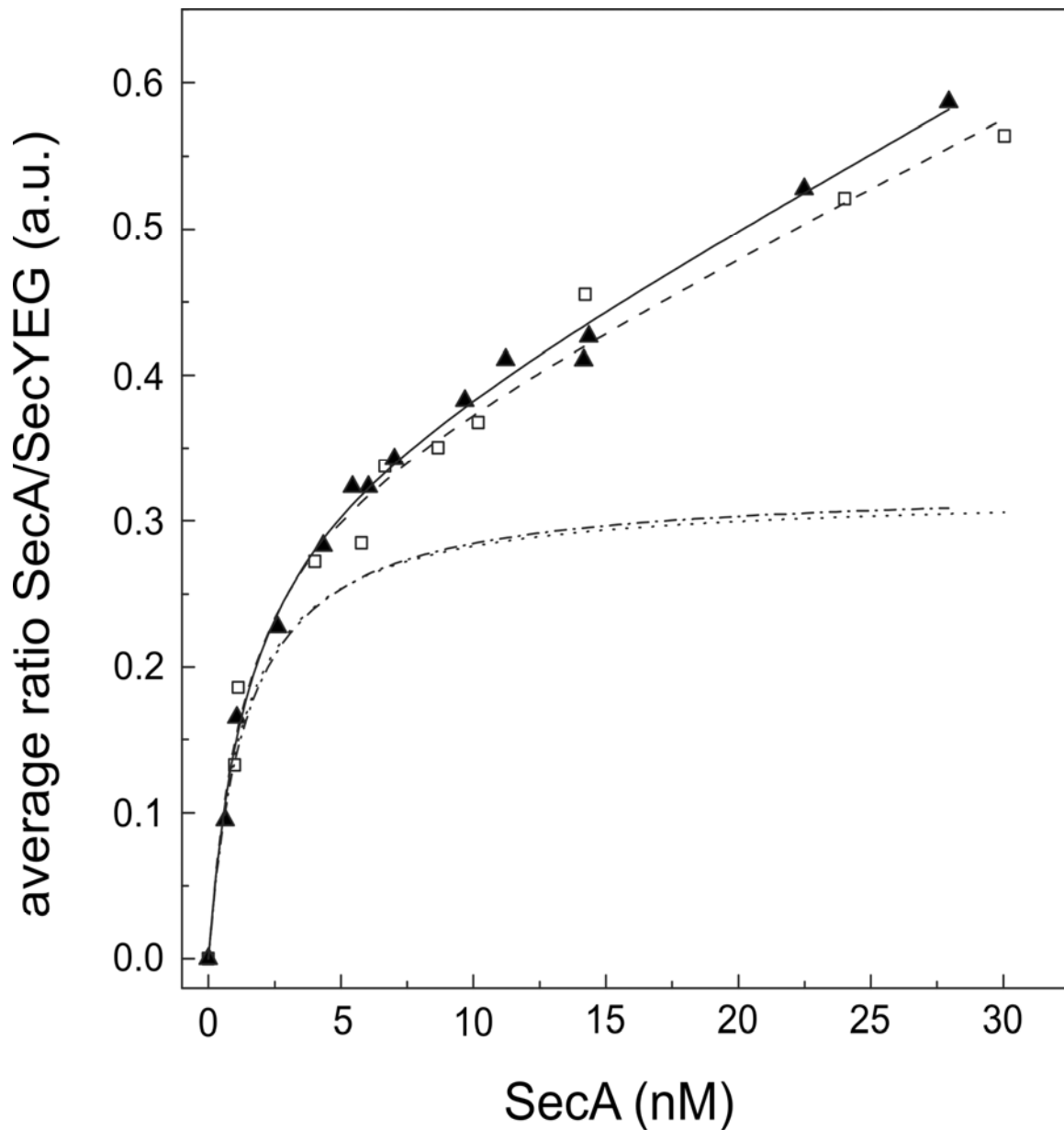


Figure S2, relates to Figure 2. Effect of proOmpA on SecA binding to SecYEG

Binding of cross-linked SecA-FM (triangles) and non cross-linked SecA-FM (squares) to SecYEG-Atto647N containing proteoliposomes in presence of proOmpA. Data points were fitted using the Hill-equation (straight and dashed lines) and the linear non-saturable binding parameter was subtracted from the fit (dotted lines).

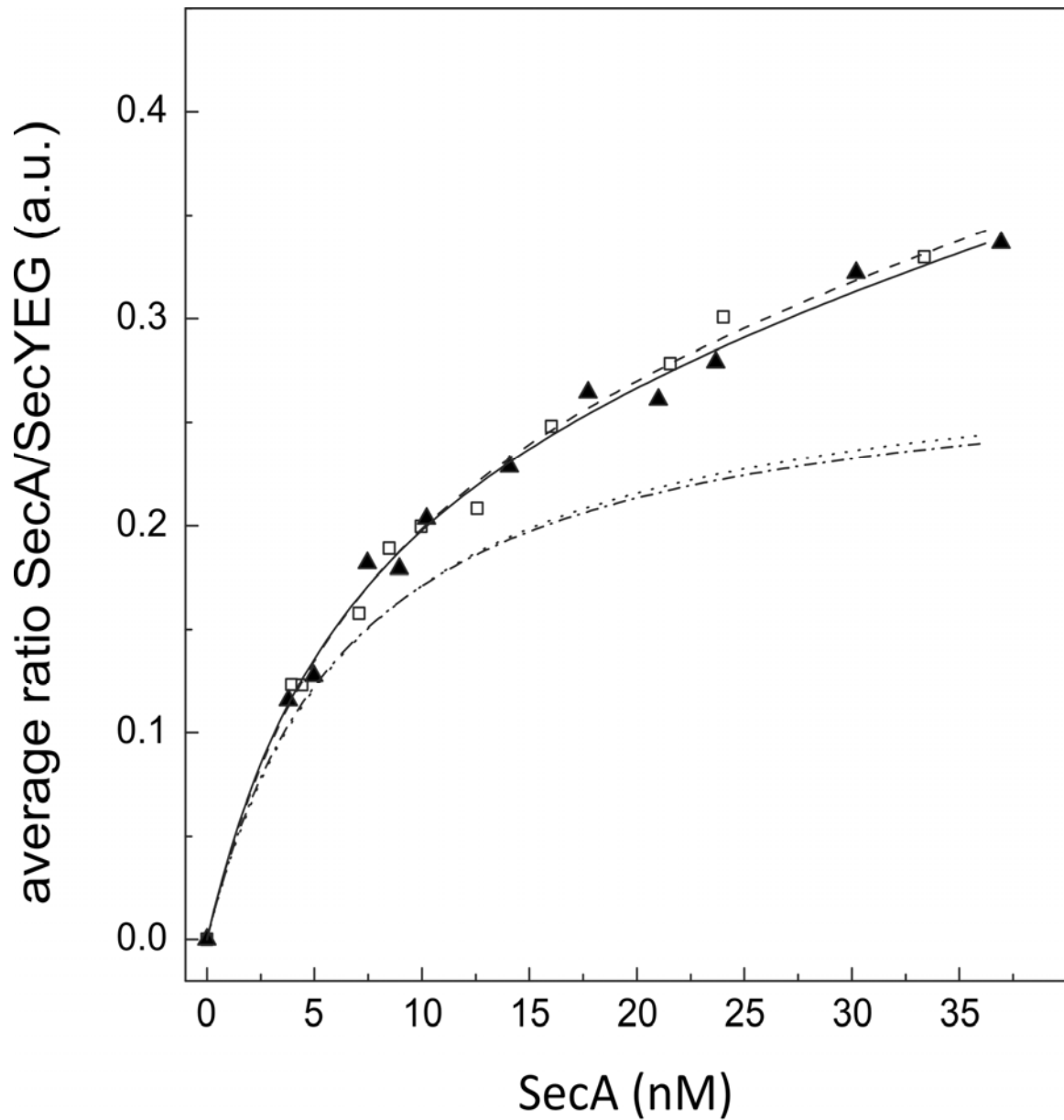


Figure S3, relates to Figure 2. Effect of ADP on SecA binding to SecYEG

Binding of cross-linked SecA-FM (triangles) and non cross-linked SecA-FM (squares) to SecYEG-Atto647N containing proteoliposomes in presence of 1 mM ADP. Data points were fitted using the Hill-equation (straight and dashed lines) and the linear non-saturable binding parameter was subtracted from the fit (dotted lines).

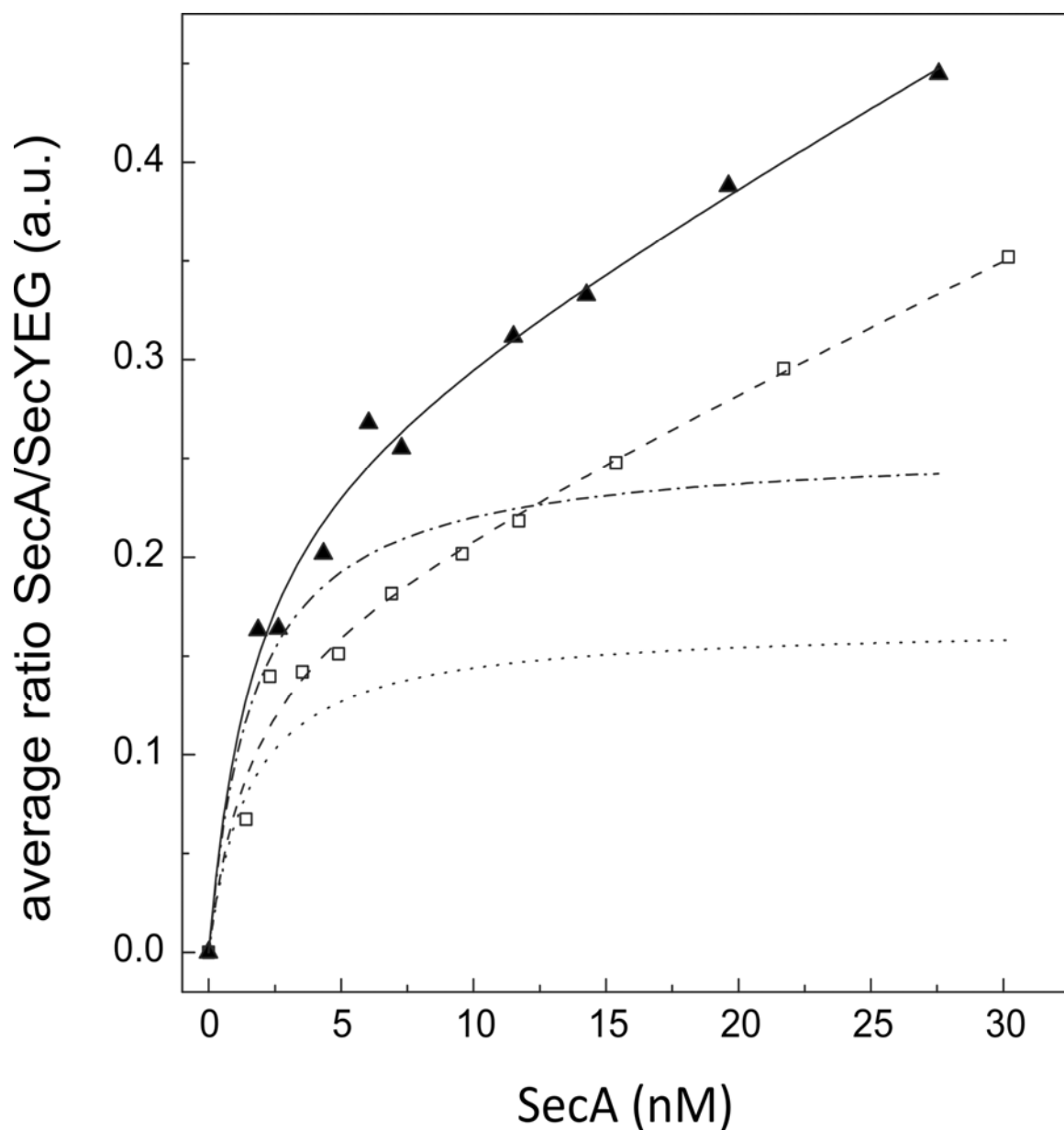


Figure S4, relates to Figure 2. Effect of urea on SecA binding to SecYEG

Binding of cross-linked SecA-FM (triangles) and non cross-linked SecA-FM (squares) to SecYEG-Atto647N containing proteoliposomes in presence of 1 M Urea. Data points were fitted using the Hill-equation (straight and dashed lines) and the linear non-saturable binding parameter was subtracted from the fit (dotted lines).

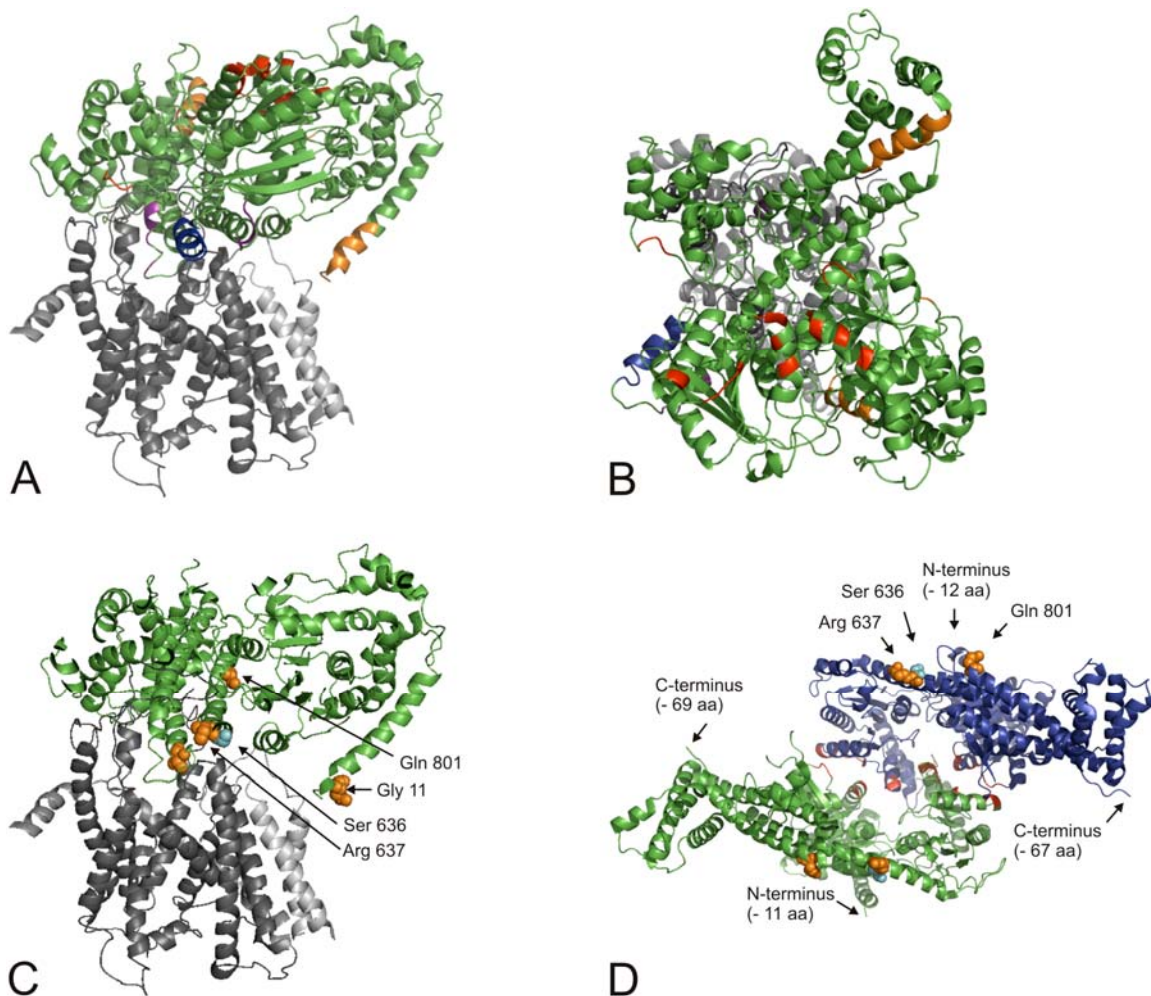


Figure S5, Relates to Figure 8. Comparison of various SecA dimerization interfaces and fit to the SecA-SecYEG crystal structure

(A) Side view of the *T. maritima* SecA-SecYEG co-crystal (Zimmer et al., 2008) with residues implicated in dimerization from dimeric SecA crystal structures from *E. coli* (red) (Papanikolau et al., 2007), *B. subtilis* (orange) (Hunt et al., 2002), *T. thermophilus* (blue) (Vassilyev et al., 2006) and *M. tuberculosis* (magenta) (Sharma et al., 2003). SecYEG is coloured in gray and SecA in green. (B) Top view (cytoplasm) of (A). (C) Side view of the *T. maritima* SecA-SecYEG co-crystal with corresponding residues found to cross-link *E. coli* SecA dimers in solution depicted as spheres. Residue numbers are from *E. coli* SecA. Orange spheres (Jilaveanu and Oliver, 2006), cyan sphere (Or and Rapoport, 2007). (D) Crystal structure of *E. coli* SecA dimer (Papanikolau et al., 2007) with residues as indicated in C). Not resolved N- and C-termini are indicated.

SUPPLEMENTAL REFERENCES

- Hunt, J.F., Weinkauff, S., Henry, L., Fak, J.J., McNicholas, P., Oliver, D.B., and Deisenhofer, J. (2002). Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. *Science* 297, 2018-2026.
- Jilaveanu, L.B., and Oliver, D. (2006). SecA dimer cross-linked at its subunit interface is functional for protein translocation. *J Bacteriol* 188, 335-338.
- Or, E., and Rapoport, T. (2007). Cross-linked SecA dimers are not functional in protein translocation. *FEBS Lett* 581, 2616-2620.
- Papanikolau, Y., Papadovasilaki, M., Ravelli, R.B., McCarthy, A.A., Cusack, S., Economou, A., and Petratos, K. (2007). Structure of dimeric SecA, the Escherichia coli preprotein translocase motor. *J Mol Biol* 366, 1545-1557.
- Sharma, V., Arockiasamy, A., Ronning, D.R., Savva, C.G., Holzenburg, A., Braunstein, M., Jacobs, W.R., Jr., and Sacchettini, J.C. (2003). Crystal structure of Mycobacterium tuberculosis SecA, a preprotein translocating ATPase. *Proc Natl Acad Sci U S A* 100, 2243-2248.
- Vassilyev, D.G., Mori, H., Vassilyeva, M.N., Tsukazaki, T., Kimura, Y., Tahirov, T.H., and Ito, K. (2006). Crystal structure of the translocation ATPase SecA from Thermus thermophilus reveals a parallel, head-to-head dimer. *J Mol Biol* 364, 248-258.
- Zimmer, J., Nam, Y., and Rapoport, T.A. (2008). Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* 455, 936-943.